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CLONING, CHARACTERIZATION, AND EXPRESSION OF ANIMAL TOXIN GENES FOR VACCINE DEVELOPMENT

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ABSTRACT

Gene libraries have been constructed from the messenger ribonucleic acid (mRNA) isolated from venom glands of different poisonous animals such as snakes, scorpions, and snails. The gene banks thus created contain recombinant clones harboring DNA sequences encoding toxins with various pharmacological activities, ranging from myonecrosis-inducing to those affecting neuronal transmission. A number of these clones have been isolated and characterized, and gene expression has been attempted

with limited success in Escherichia coli, baculovirus, and in two mammalian cell expression systems by using either cDNAs or synthetically-constructed genes.

INTRODUCTION

The advent of molecular immunology and recombinant DNA technology has engendered research in every area of the biological sciences. The role played by these technologies in vaccine development is becoming increasingly pivotal with the emergence of synthetic peptides; anti-idiotypes; and genetically engineered, live, attenuated recombinant viruses as potential vaccines. Vaccination can afford protection against toxic proteins by presenting relatively harmless antigens to the immune system, thereby allowing the body to establish adequate levels of antibody and inducing an anamnestic response in which a primed population of cells can grow when the antigen reappears in its virulent form. Through the development and use of vaccines, many bacterial and viral diseases have been brought under control. However, there are many animals that produce potent neurotoxins and membrane-damaging toxins whose effects are acutely deleterious to man. Basic research studies and vaccine development against many animal toxins has been slow for economical reasons. To date, investigative efforts in these areas have focused on the elucidation of structure-activity relationships of several animal toxins and the production of non-toxic antigens that could be used as vaccine candidates against these toxins.

Recombinant DNA technology has been used to clone and characterize animal toxin genes encoding proteins with variable pharmacological activities. Current endeavors have concentrated initially on the expression of cloned genes in host systems that can process the recombinant products into biologically active forms. After suitable hosts are obtained for the expression of toxin genes, site-directed mutagenesis may be employed as a means of altering the cloned genes in specific regions so the resulting recombinant products will have strategically replaced amino acid residues relative to the native toxin. Alteration of designated amino acid residues in the protein via gene modification can lead to the identification and modification of amino acids responsible for toxic activities. Future research goals will involve the generation of cross-reacting materials (CRMs), which are mutant proteins that have lost their toxic or functional activities while still retaining immunogenicity. This research will yield insights into the structure-activity relationships of animal

toxins and generate potential vaccine candidates against these same toxins.

Although the application of molecular genetics to animal toxin research has been slow to develop, many investigators without formal training in these techniques are now discerning the potential usefulness offered by recombinant DNA technology. I hope this treatise will be of special benefit to them. The purpose of this review is to present a brief background on animal protein toxins; survey recombinant DNA techniques directly applicable to the cloning, characterization, and expression of genes encoding animal toxins; and review published and ongoing work on the molecular biology of animal toxin genes.

CLASSES OF ANIMAL TOXINS

Many different kinds of animal protein toxins exist in nature. The sources from which they originate are equally as diverse. They range from certain types of marine snails (1-3); stinging coelenterates (4) and fish (5); to spiders (6-9), beetles (10), scorpions (11-13), snakes (14-17), insects (18-20), and even frogs (21). There have been numerous studies on many of these toxins, that deal with their purification, characterization, and mechanism(s) of action. For protein toxins, characterization may include determinations of molecular weight and subunit structure; elucidation of the amino acid sequence via Edman degradation and mass spectroscopy; ascertainment of the lethal potency of the toxin(s) by bioassays in animal models; and, in some cases, conformational analysis by techniques such as NMR spectroscopy and X-ray diffraction. Mechanism of action studies help define the pharmacological effects of a highly purified toxin species in vivo and in vitro, the location or the site of binding as well as the affinity of the binding to a cell receptor, and the relationship of any biochemical activity the toxin may possess to its toxicity. The above studies have been extensive with snake, scorpion, and snail toxins, and many reviews have been written on these subjects (22-30).

The pharmacological effects of these purified venom components include neurotoxic, membrane-damaging, and blood coagulation/anti-coagulation-inducing effects. For example, various components of animal venoms exert their coagulant activity at various levels of the blood-clotting cascade, as in the case of the thrombin-like enzymes found in snake venoms. Membrane-damaging toxins can be cytolysins that produce hemorrhage in tissues and are destructive to endothelial cells and blood

sels (hemorrhagins), or they can be hemolysins assaulting erythrocytes and interrupting their structural integrity resulting in the liberation of hemoglobin from red blood corpuscles. Neurotoxins are substances that

TABLE 1

PROTEIN TOXINS FROM ANIMAL ORIGIN

I. ION-CHANNEL TOXINS
  A. SODIUM- CHANNEL TOXINS
    1. Myotoxin a (prairie rattlesnake, Crotalus viridis viridis) (31)
    2. Alpha scorpion toxins (Old World scorpions; fat-tailed scorpions, Androctonus) (32)
    3. Beta scorpion toxins (New World scorpions; bark scorpions, Centuroides) (33)
    4. Sea anenome toxin (European stinging anemone, Anemonia sulcata) (34)
    5. Mu conotoxins (geographer cone snail, Conus geographus) (35)
  B. POTASSIUM-CHANNEL TOXINS
    1. Apamin (honey bee, Apis mellifera) (36)
    2. Dendrotoxin (Eastern green mamba snake, Dendroaspis angusticeps) (37)
    3. Charybdotoxin (Palestine yellow scorpion, Leiurus quinquestriatus hebraeus) (38)
    4. Noxiustoxin (a Mexican bark scorpion, Centuroides noxius) (39)
  C. CALCIUM- CHANNEL TOXINS
    1. Omega conotoxins (geographer cone snail, Conus geographus) (35)
    2. Toxins AG1 and AG2 (American funnel-web spider, Agelenopsis aperta) (40,41)
II. PRESYNAPTICALLY-ACTING TOXINS
  A. Snake venom phospholipases
    1. Notexin (Australian tiger snake, Notechis scutatus scutatus) (42)
    2. Mojave toxin (Mojave rattlesnake, Crotalus scutulatus scutulatus) 43)
    3. Beta-bungarotoxin (banded krait snake, Bungarus multicinctus) (44,45)
    4. Taipoxin (Australian taipan snake, Oxyuranus scutellatus) (45,46)
III. POSTSYNAPTICALLY-ACTING TOXINS
    1. Erabutoxin a (erabu sea snake, Laticauda semifasciata) (47,48)
    2. Alpha-cobratoxin (Thai cobra snake, Naja naja kaouthia) (47,49)
    3. Alpha-conotoxins (geographer cone snail, Conus geographus) (35)
IV. MEMBRANE-DAMAGING TOXINS
    1. Hemolysins (cardiotoxins from the Formosan cobra snake, Naja naja atra) (50,51)
    2. Hemorrhagins (habu snake, Trimeresurus flavoviridis) (52)
    3. Myotoxin a (prairie rattlesnake, Crotalus viridis viridis) (31)
V. COAGULATION/ANTI-COAGULATION TOXINS
    1. Ancrod (Malayan pit viper snake, Calloselasma rhodostoma) (53,54)
    2. Batroxobin (terciopelo snake, Bothrops atrox) (53,55)
    3. Echistatin (carpet viper, Echis carinatus) (53,56)
VI. MISCELLANEOUS TOXINS
  A. IONOPHORES
    1. Alpha-latrotoxin (black widow spider, Latrodectus mactans) (57)
    2. Diamphotoxin (chrysomelid beetle, Diamphidia nigro-ornata) (10)
  B. ANTI-CHOLINESTERASE TOXINS
    1. Fasciculin (Eastern green mamba snake, Dendroaspis angusticeps) (58)

affect the normal functioning of excitable tissues attributable to a specific recognition and binding affinity to distinct sites in these tissues. Neurotoxins can be categorized according to effects and sites of action in the nervous system. For example, ion-channel toxins modify ion conductance, presynaptic toxins affect neurotransmitter release, and postsynaptic toxins interfere with the binding and resulting action of neurotransmitters. Table 1 presents a list of the various classes of animal toxins with specific examples for each group and the animal source from which the toxin was derived. Most of the molecular biology research that has been performed with animal toxins has pursued the study of snake, scorpion, and snail neurotoxins. The focus of this review will be on the genes encoding these toxins.

PROTEIN STRUCTURE OF ANIMAL TOXINS

One of the most intriguing queries some herpetologists ask is, "how did it come about that snakes manufacture such lethal toxins?" Some questions have been posed regarding whether or not venom evolution has contributed to increased fitness for the ophidian species equipped with these formidable substances. The queries are quite challenging considering that many venomous and non-venomous species appear equally successful (59). Yet herpetologists present detailed and convincing explanations why some snakes did evolve venom glands for survival competition (23). If the enigmatic evolutionary nature of snake venoms is captivating to naturalists, then the structural and functional diversity of purified toxins should be as interesting to the protein chemist. Many of the presynaptically-acting neurotoxins isolated from snake venoms thus far have protein structures that vary greatly, as shown in Table II. Irregardless of the differences in protein structure among the above toxins, all possess phospholipase A2 (PLA2) activity, function at the prejunctional neuromuscular level, and ultimately cause the inhibition of acetylcholine release from presynaptic nerve terminals. In addition to structural diversity, many of the snake venom neurotoxins exhibiting PLA2 activity possess varying degrees of lethal potency in mice, as well as differences in enzymatic activity. Extensive research on the relationship between PLA2 activity and toxicity has not fully clarified the confusion among toxinologists, which still pervades this association. It is possible that the use of molecular biology can be of some help in answering this question in the future. The structural heterogeneity among neurotoxins with similar or identical functions is not restricted to snake venom presynap-

TABLE II

SNAKE PRESYNAPTICALLY-ACTING NEUROTOXINS

NEUROTOXIN	*MW	#AA	#S-S	#SUBUNITS	**POTENCY	REF.
1. Notexin	13,600	119	7	1	17	60
2. Notechis II-1	13,600	119	7	1	0	61
3. Notechis II-5	13,600	119	7	1	45	62
4. Pseudexin A	16,660	117	7	1	1300	63
5. Pseudexin B	16,660	117	7	1	750	63
6. Pseudexin C	16,700	117	7	1	0	63
7. Ammodytoxin A	14,000	122	7	1	20	64
8. Ammodytoxin B	14,000	122	7	1	580	65
9. Ammodytoxin C	14,000	122	7	1	360	66
10. β-bungarotoxin	24,000	208	14	2	14	67,68
11. Crotoxin	24,000	208	14	2	50	69
12. Mojave toxin	24,000	208	14	2	50	70
13. Taipoxin	45,000	375	22	3	2	71
14. Textilotoxin	80,000	-	-	5	1	72

* MW = approximate molecular weight

** potency = μg/kg i.p. in mice to induce lethality

TABLE III

POSTSYNAPTICALLY-ACTING NEUROTOXINS

NEUROTOXIN	*MW	#AA	#S-S	#SUBUNIT	**POTENCY	REF.
1. Conotoxin G1	1450	13	3	1	1	35
2. Erabutoxin a	6850	62	4	1	150	48
3. α-bungarotoxin	7980	74	5	1	139	74,75

SODIUM-CHANNEL NEUROTOXINS

NEUROTOXIN	*MW	#AA	#S-S	#SUBUNIT	**POTENCY	REF.
1. Mu-conotoxin	2300	22	3	1	-	35
2. AaH II scorpion toxin	6800	64	4	1	10	73,76
3. Sea anemone ATX-II	4935	47	3	1	-	77

* MW = approximate molecular weight

** potency = μg/kg i.p. in mice to induce lethality

tic neurotoxins. These differences are also observed with ion-channel and postsynaptic neurotoxins as well, as shown in Table III. Although animal toxins are relatively low molecular weight proteins, their protein structures are, nevertheless, complex. Most have multiple disulfide bonds and many have up to 14 disulfide bridges. Some have modifications at their

carboxyl-terminus as in the case of many conotoxins (35), scorpion toxins (73) and bee venom toxins (36). This modification consists of a C-terminal α-amide group (-X-NH_2). About half of the bioactive peptides found in nervous and endocrine systems possess a C-terminal α-amide moiety (78), and for most of these, that modification is important to bioactivity (79). It is noteworthy to point out that the C-terminally modified toxins from marine snails, scorpions, and honey bees are secreted products from exocrine glands. Although many toxins are secreted from snake exocrine glands, snake toxins are not known to possess C-terminal amidations, although there is evidence to suggest some snake mRNAs may encode precursor proteins capable of having this modification.

Other toxins have their amino-terminal residues blocked. Several examples include, dendrotoxin and toxin I (37,80), chains B and C from the acidic subunit of crotoxin (81,82), and chain C from the acidic subunit of Mojave toxin (83), in which the N-terminal amino acids have been modified to pyroglutamate. The function of this modification is not known. There are additional venom components that are glycosylated, such as the γ-subunit of taipoxin (71). Many snake venom presynaptic neurotoxins possess quaternary complexes (69-72) which are required if maximum lethal potency of the toxin is to be achieved. Finally, as stated above, these toxins are secreted as exocrine products, and as such, are generally synthesized with an N-terminal signal sequence necessary for initiating the exportation process from the cell (84). Usually, the signal peptide must be removed if the protein is to achieve its biologically active conformation. As with other protein modifications, removal of the signal sequence occurs as a cellular post-translation processing event. In order to express these toxins, one must be capable of manipulating the cloned gene and its host or its expressed recombinant product in such a fashion as to reproduce the processing functions as they occur in the animal. The next section will be devoted to the techniques that can be used in performing this formidable task.

RECOMBINANT DNA TECHNOLOGY

There are many publications describing in detail various techniques to be used in creating gene libraries; for screening gene banks in search of recombinant isolates of interest; and characterizing cloned inserts by sizing, mapping, and nucleotide sequence analysis (85-88). Without prior background on the theory and application of recombinant DNA techniques, a novice can be intimidated and confused by some of the literature on these

subjects. Simply explained, genes can be cloned either from the genome, as reverse transcripts from messenger ribonucleic acid (mRNA), or as synthetic constructs from organic chemical reactions. Genes are cloned from the genome by extracting and purifying the deoxyribonucleic acid (DNA) from tissues (e.g., liver, heart, pancreas) of the species of interest. Extraction procedures usually involve the use of phenol/chloroform mixtures and the DNA can be concentrated and further purified by precipitation with ethanol. The next step is to fragment the purified DNA, which can be achieved by using restriction endonucleases that recognize and enzymatically cleave specific deoxynucleotide sequences in the DNA called palindromes. As is the case with all cloning methods, a vector or vehicle subsequently is required to transport the genes or DNA fragments into a host (e.g., E. coli), which will amplify the recombinant vector. Vectors for cloning are plasmids or phage lambda DNA or combinations of both. There are different types of plasmid and phage DNA vectors (89-92), each having various features to assist the researcher in cloning, screening, characterization, and subsequent expression of the cloned gene. Some of these characteristics include multiple cloning sites, selectable markers, primers flanking unique restriction enzyme cloning sites for future dideoxy-sequencing, and inducible promoters for expression in prokaryotic and eukaryotic organisms.

The type of vector to be used for the genomic library construction depends to a great extent on the expected size of the genes to be cloned. Since animal toxins have low molecular weights, it is likely that the genomic precursors encoding the toxin mRNAs will be relatively low molecular weight even with their regulatory and presumed intronic sequences present. Lambda vectors, such as ZAP (93,94) and GT11 (95) are efficient for cloning DNA fragments with sizes of 10 kilobases (Kb) and smaller, and thus can be suitable vectors for producing genomic libraries. Since multiple variations exist for linking genomic fragments to vectors, only one example is presented here. Assuming that lambda ZAP is chosen as the vector, the DNA extracted from the animal tissue as well as lambda ZAP itself is separately cleaved by the restriction enzyme, EcoR 1. In some cases, non-recombinant background isolates can be reduced by also treating the lambda vector with bacterial alkaline phosphatase before enzymatically joining the DNA fragments to the vector with T4 DNA ligase. This treatment inhibits the vector from ligating back on itself and becoming a transformable species. After the DNA fragments have been ligated to the vector, the recombinant constructs can be packaged into viable phage particles by using commercially available bacterial packaging extracts.

In-vitro packaging is an efficient means of introducing recombinant lambda into host cells (85-87). Infection of E coli with recombinant lambda generates primary genomic libraries ready to be analyzed for the presence of clones harboring genes of interest. Screening strategies will be considered subsequent to a discussion on cDNA library constructions.

All procedures used to construct cDNA libraries require the extraction and purification of mRNA from cells in which the most abundant amount of poly(A)$^+$ RNA is expected to be found. In the case of mRNA encoding exocrine-secreted toxins, the glands themselves are the source of the mRNA. Since geographical variation of venom proteins among snakes of the same species has been documented (96), source and origin of snakes used for gene construction can be important. Venom samples from individual animals to be used for molecular biology studies should be analyzed for the presence and quantity of individual constituents of interest prior to the removal of tissues from the animal. In addition, before removing glands from animals, it is desirable to evacuate the venom gland(s) 1 day prior to their removal in order to increase the potential yield of poly(A)$^+$ RNA (97). Glands removed should be frozen in liquid nitrogen and stored at -70^{0}C until used. The key for making utile cDNA libraries is to start with intact, purified mRNA. This can be accomplished by minimizing ribonuclease activity from the cells or tissues during the initial stages of extraction and exercising added precautions to avoid introduction of ribonuclease contaminations from glassware and solutions (85). One of the most efficient methods for extracting total RNA from cells or tissues is the guanidinium isothiocyanate-hot phenol extraction method (98). Total RNA from this extraction is then subjected to oligo-dT column chromatography to enrich the mRNA pool (99). The integrity of the poly(A)$^+$ RNA and its ability to serve as a template for full-length cDNA transcription can be evaluated on a small scale prior to library-sized reactions. Many commercial cDNA kits are available which contain buffers, dNTP solutions, oligo-d(T) primer, purified enzymes necessary for cDNA synthesis, and detailed manuals explaining each step.

Various methods exist for designing cDNA libraries. Essentially all the methods for constructing cDNA rely on a primer-initiated reverse transcriptase to create the complement of the mRNA sequences. The newly formed cDNA is then used as a template to synthesize a double-stranded DNA corresponding to the sequence of the mRNA. For molecular cloning, the duplex cDNAs are covalently linked to a plasmid or bacteriophage vector via complementary homopolymeric tailing or cohesive ends created with

linker or adaptor segments containing appropriate restriction sites. Two different cDNA cloning schemes are presented in Fig. 1A and 1B. In the first method (100) (Fig. 1A), after the double-stranded cDNA is completely synthesized, it is joined to the vector; while in the Okayama/Berg system (101,102) (Fig.1B), the mRNAs are annealed to a vector at the outset and cDNA synthesis occurs while the DNA is attached to the vector. In both procedures, reverse transcriptase is used to synthesize the primary strand and ribonuclease H is subsequently employed to remove the mRNA while simultaneously preparing efficient primers for DNA polymerase to complete the second-strand synthesis. The first method has commercially available adaptors or linkers and T4 DNA ligase to fuse the cDNAs with appropriate vectors (Fig. 1A). Subsequently, the newly formed recombinant plasmids are used to transform E. coli while recombinant lambda vectors are packaged into viable phage particles prior to infecting E. coli.

The Okayama/Berg strategy utilizes terminal deoxynucleotidyl transferase to produce 3'-terminal d(C) extensions on the linear recombinant vector (pcDV-1) and a d(G) tail on one end of a linker fragment (PI-1). After a Hind III site is created in the recombinant pcDV-1 vector, the linker is used to join and cyclize the 5'-end of the cDNA with the opposite end of the expression vector. After the second strand synthesis is complete and the linker fragment has been covalently sealed with T4 DNA ligase, the recombinant molecules are used to infect E. coli. The pcDV-1 plasmid contains SV40 polyadenylation sequences; and the linker fragment, PI-1, contains the SV40 origin of replication, early region transcription promoter, and mRNA splicing sequences. These additional sequences create a recombinant whose cDNA insert can be transcribed and processed in mammalian cells and, if the cDNA contains the entire protein coding sequence, can direct the production of the relevant protein (102).

The primary or amplified libraries are now ready for screening. Libraries or individual clones can be examined for their insert content by using appropriately radiolabeled nucleotide probes with sequence homology to a portion of the desired gene. There are other techniques available for screening gene libraries, such as immunological methods. However, immunological methods rely on the assumption that the cloned gene is expressed, the product solubilized, and in a conformational state that will be recognized by existing antibody reagents. As discussed above, the post-translational modifications necessary to promote the formation of biologically active toxins are complex and E. coli may not have the capability of performing those tasks. As the amino acid sequences for many animal toxins are available, the most efficient screening procedure for libraries

containing animal toxin genes is hybridization with isotopically labeled oligonucleotide probes generated from known protein sequences or cDNA probes, if they are available. DNA from recombinant bacterial colonies or plaques that hybridize to isotopically tagged probes can be further characterized by restriction mapping, insert size determination, and DNA sequence analysis.

Aside from expressing the gene and characterizing the recombinant product, DNA sequence analysis is the only method for unequivocally determining whether or not a positive clone from a library screen actually contains the desired gene. There are two different approaches to sequencing DNA: one is the Maxam and Gilbert method which is chemistry based (103), while the second, Sanger or dideoxy chain termination sequencing, is enzymatic (104). Deoxynucleotide sequencing kits containing reagents and instructions for both types of sequencing are commercially available. Dideoxy chain termination has become extremely popular with the construction of cloning vectors, such as M13 (105), pGEM (106), and Bluescript (94), that contain unique priming sequences for the polymerizing enzymes (e.g., Klenow, Taq DNA polymerase) used in this method. Inserts from positive library clones can be subcloned into these vectors by restriction enzyme excision of the insert from the DNA clone and reinserted into the specialized vector (85) or, in some cases, by using an in-vivo homologous recombination procedure (94). DNA fragments cloned into plasmid or M13 vectors are frequently longer than 400 bases and thus may be too long to sequence from a single primer binding site on the vector. In those cases, synthetic primers can be prepared from previously determined sequences, or a nested set of deletions in the target DNA can be generated by using exonuclease III and S1 nuclease (107), both of which effectively advance the priming site nearer the sequence of interest.

After the nucleotide sequence of the cloned gene has been determined, ensuing experiments usually include expression of the cloned gene. There are a number of different expression systems available for gene expression. The choice of system to be used depends somewhat on the genes to be expressed. Table IV lists some of the various hosts for expresssing genes. Prokaryotic hosts (e.g., E. coli) have been used to express many different kinds of heterologous genes as fused or unfused products (108). In addition, expression of cloned genes in E. coli can be regulated by thermal (109) or chemical (110) induction depending on the vector used. Sometimes, however, prokaryotic hosts express foreign genes poorly or their expressed products are biologically inactive. In addition, bacterial cells do not carry out post-translational modifications such as glycosylation,

amidation, phosphorylation, and cleavage of protein precursors. For these reasons, progress in molecular genetics has relied partly upon the availability of a broad and increasingly sophisticated array of cloning vectors and host systems.

TABLE IV

HOSTS FOR EXPRESSING GENES

1. PROKARYOTIC (e.g., E. coli, Bacillus subtilis)
2. YEASTS (e.g., S. cerevisiae)
3. VIRUSES
   a. Papilloma virus
   b. Vaccinia virus
   c. Baculovirus
4. MAMMALIAN CELLS
   a. Monkey kidney COS cells
   b. Chinese hamster ovary cells (CHO)

Saccharomyces cerevisiae or Bakers' yeast is another convenient host for the expression of heterologous genes. Benefits of using yeasts include high levels of secretion, the ability to express more protein per liter than mammalian systems although less than E. coli, and no additional protein refolding steps required which may be necessary in bacterial expression systems. One family of yeast expression vectors (111) was developed, which features plasmid replication and antibiotic selection in E. coli, packaging of single-stranded DNA upon infection of E. coli with filamentous helper phage, replication in S. cerevisiae based on the 2 μm plasmid origin of replication, and selection in yeast by complementation of LEU2 or URA3 genes. Unique restriction enzyme cloning sites are also available within an "expression cassette," which includes the promoter and 3' sequence of the ADH1 gene. These vectors can be used for cloning, expression in yeast, sequencing, and mutagenesis without the need to be recloned into other vectors.

Viral (112,113) and mammalian (114,115) expression systems have also supplemented the genetic engineer's "biological tool chest." Because of their potential use in vaccine development, certain eukaryotic viruses, such as vaccinia virus (112) and baculovirus (113), have recently become popular as foreign gene expression systems and expression vectors. Vaccinia virus has been developed as a live, infectious expression vector for foreign genes inserted into the viral thymidine kinase gene (116). Insertion of a foreign gene into vaccinia virus can create a eukaryotic re-

combinant vector capable of expression in an in vivo animal model. In contrast to in vivo expression, the helper-independent baculovirus expression vector has been used to express a wide variety of heterologous genes in cell culture. Baculovirus vectors have achieved widespread acceptance for their ability to express proteins of agricultural and medicinal importance. A baculovirus vector was used to express the first recombinant HIV envelope proteins to receive Food and Drug Administration approval for clinical evaluation as a vaccine candidate for the acquired immunodeficiency syndrome (117). Autographa californica nuclear polyhedrosis virus is an insect virus (baculovirus) which can express foreign genes to remarkably high levels when a foreign gene is inserted into the modified polyhedrin gene promoter of the virus (113,118). One of the major advantages of this invertebrate viral expression vector over bacterial, yeast, and mammalian expression systems is the abundant expression of recombinant proteins, which are, in many cases, structurally and functionally similar to the authentic gene products. In addition, baculovirus is not pathogenic to vertebrates or plants and does not employ transformed cells or transforming elements, as do the mammalian expression systems. The baculovirus vector also utilizes many of the protein modification, processing, and transport systems that occur in higher eukaryotic cells and may be essential for the complete biological function of a recombinant protein (113,118).

A wide assortment of established cell lines and DNA vectors have been used to express cloned genes in mammalian cells. Some vector-cell systems, such as the Simian virus 40 (SV40) vectors in monkey kidney (COS) cells (102) have been developed for high levels of transient expression. Transient expression systems are valuable for determining rapidly if cloned genes or cDNAs can be expressed and whether or not the recombinant product has been processed properly. Production of larger quantities of protein from cloned genes or cDNAs, however, requires prolonged expression of the genes in stable cell lines. Thus, vector-cell systems have been developed for stable, long-term expression in mammalian cells. High-level expression in stable cell lines is based on the concept, that if foreign-gene copy number is elevated in each cell, this will lead to higher levels of protein production in those cells. Two methods of achieving high-gene copy number, and, therefore, high production levels, have been developed. In one method, the copy number of the recombinant gene is elevated by co-amplification of the number of desired protein-encoding genes with that of a selectable, amplifiable gene. Such a system based on co-amplification uses vectors containing the gene encoding dihydrofolate reductase as the selectable marker and Chinese hamster ovary (CHO) cells as

the host cell line (119). The second method relies on viral DNA replication to achieve high-copy number of the cloned gene. Mouse C127 fibroblast cells can be infected with bovine papilloma DNA vectors containing genes of interest. These recombinants can replicate and are often maintained in the cells episomally or are tandemly integrated into the mouse chromosomal DNA (120). Both methods serve to increase the copy number of the gene to be expressed. By coupling the protein-encoding sequences to strong promoters and enhancer regions, a high level of transcription can subsequently be achieved (121).

MOLECULAR BIOLOGY OF ANIMAL TOXINS

To date, only a limited number of snake venom toxin genes have been cloned and characterized. These cloned genes encode for pre- and postsynaptic neurotoxins, membrane-damaging toxins, a procoagulant snake venom toxin, and an anti-cholinesterase toxin. The first cloned and sequenced cDNA encoding a snake venom protein with sequence homology to known PLA2 enzymes was from the black-banded sea snake, Laticauda laticaudata, captured in New Caledonia (122). This full-copy gene specified a pre-protein with a 27-amino acid signal peptide and an 118-amino acid structural protein. Since that work, other cDNAs encoding snake venom PLA2 homologs have been cloned from the olive sea snake, Aipysurus laevis, collected at the Great Barrier Reef in Australia (123); from the tiger snake, Notechis scutatus scutatus, captured near Sidney, Australia (124); from the South American rattlesnake, Crotalus durissus terrificus (125); from the European long-nosed viper, Vipera ammodytes ammodytes, collected around Slunj, Croatia (126); and from the Mojave rattlesnake, Crotalus scutulatus scutulatus, collected near Portal, Arizona (127). The cDNA clone isolated from the Aipysurus laevis cDNA library (123) encoded a precursor phospholipase A2 with a 27-amino acid signal peptide and an117-amino acid structural protein. Interestingly, there had been no reports of phospholipase A2 enzymes in the venom of this snake. The nucleotide sequence reported for a cDNA isolate from a Notechis scutatus scutatus cDNA library also encoded a preprotein with a signal peptide of 27 residues and a structural protein of 118 amino acids. Similar to the Aipysurus laevis PLA2, the inferred sequence from the Notechis cDNA encodes for another PLA2 homolog that has not been reported from this snake venom. However, the amino acid sequence resembles a closely related protein in the venom called Notechis II-1, which has both low toxicity and PLA2 activity (124). All of the above cDNAs had 5' and 3' untranslated

regions which were found to be largely conserved. The cDNAs from Laticauda and Notechis had putative polyadenylation sequences on their 3' end while the cDNA from Aipysurus was lacking this sequence.

Many of the phospholipase A2 enzymes isolated from snake venoms produce varying degrees of neurotoxicity in animals (128), while some are non-toxic. The cDNAs cloned from the European long-nosed viper, and the South American and Mojave rattlesnakes encode snake venom phospholipase A2 enzymes known to be relatively potent presynaptic neurotoxins (64,69,70). Clones have been isolated from a Vipera ammodytes ammodytes cDNA library that encode for ammodytoxins A, B, C and two non-toxic PLA2s (129); one of these, ammodytoxin C, has been characterized (126). The ammodytoxin C cDNA encodes a precursor protein with a 16-amino acid signal peptide and a 122-amino acid structural protein. Two other presynaptic phospholipase neurotoxins, crotoxin and Mojave toxin, are each heterodimers containing two non-covalently complexed subunits. One subunit is a basic, single-chained protein with PLA2 activity and low toxicity, while the other is acidic, non-toxic, lacks PLA2 activity, and is actually composed of three peptide fragments held together by seven disulfide bonds. The amino acid sequence of the basic and acidic subunits of crotoxin and Mojave toxin have been determined (81,82, 83,130,131). Two cDNAs encoding the two subunits comprising crotoxin have been isolated from a Crotalus durissus terrificus cDNA library and sequenced (125). The cDNA for the basic subunit encodes a precursor protein with a 16-amino acid signal peptide and a 122-amino acid structural protein, which differs from the published amino acid sequence only at position 65, in which proline replaces arginine. The cDNA encoding the acidic subunit specifies the same 16-amino acid signal peptide found in the basic subunit cDNA and a 122-amino acid structural protein with the same sequence as that reported for the acidic subunit of crotoxin. Both of the cDNAs described had long, 5' untranslated sequences but lack a putative polyadenylation signal on the 3' end, suggesting that part of the 3' terminus may have been removed at some point during library construction or clone characterization. It is interesting that the signal peptides from crotoxin (125) and ammodytoxin C (126) have an 81% homology, while the mature crotoxin basic subunit has only a 59% sequence homology with ammodytoxin C.

A Mojave rattlesnake cDNA library was constructed (127) and cDNAs encoding the basic and acidic subunits isolated and sequenced. The cDNA for the basic subunit encodes a precursor protein with a 16-amino acid signal peptide identical to the signal peptide for crotoxin (125) and a 122-amino acid structural protein identical to the amino acid sequence

determined for the Mojave toxin basic subunit (131). There were many clones in the library that had insert sizes of approximately 650 base pairs encoding the Mojave toxin acidic subunit, in agreement with that published for the crotoxin acidic subunit gene (125). However, one of the most notable isolates characterized from this library was a 1.9-Kb cDNA encoding the acidic subunit for Mojave toxin. This Mojave toxin acidic subunit cDNA, designated Css-M6, had five regions with open reading frames (ORF) beginning with ATG and having termination signals downstream. While the function of the first four ORFs (ORF A, B, C, and D) are unknown at the present time, ORF region E (Fig. 2) encoded the acidic subunit peptides. The inferred amino acid sequence from this region was identical to that presented for the acidic subunit of crotoxin (81,82), except at positions 62 (glutamic acid for phenylalanine), 74 (aspartic acid for asparagine), 93 (glycine for glutamic acid), 114 (aspartic acid for asparagine), 117 (asparagine for aspartic acid), and 125 (arginine for glutamine). These differences are in agreement with those derived from the direct sequencing of the Mojave acidic subunit (83). Based on the sequence for the cDNAs encoding the basic and acidic subunits of the crotoxin and Mojave toxin precursors (125,127), it is presumed that the signal peptide is 16 amino acids long, as this sequence is identical for both subunits and divergence begins subsequent to this sequence. Thus, within the sequence following the putative signal peptide (Fig. 2), three additional regions exist where post-translational processing occurs, based on the known amino acid sequence for the acidic subunits of crotoxin and Mojave toxin. For Mojave toxin acidic subunit, three regions are proteolytically removed during processing to form the A chain, B chain, and C chain(s). These regions are from residues 17 to 40, 81 to 83, and 120 to 126 or 120 to128, respectively (Fig. 2). Two isoforms of the acidic subunit C chain were sequenced by direct sequence analysis (83). As a result of protease cleavage within the interior of the proteins, glutamine residues at the amino-termini of the the B chain in Mojave toxin (83) and in the B and C chains of crotoxin (81,82) are converted into pyrrolidone carboxylyl derivatives. The acidic subunit of crotoxin and Mojave toxin enhance the pharmacological efficacy and, in particular, the lethal potency of the PLA2-containing basic subunit presumably by acting as the receptor-binding domain of the toxins.

The first snake venom postsynaptic neurotoxin cloned and sequenced was erabutoxin a, from the erabu sea snake Laticauda semifasciata (132). Since that original work, the cDNA encoding erabutoxin b, which also blocks the nicotinic acetylcholine receptor, has also been cloned and sequenced (133). Both cDNAs encode precursor proteins with signal peptides

of 21-amino acids and structural proteins of 62-amino acid residues. The two cDNAs have exactly the same nucleotide sequence except for one base change, resulting in a single amino acid substitution at position 26 for erabutoxin b (asparagine for histidine). The Aipysurus laevis cDNA library previously noted was also screened for genes encoding postsynaptic neurotoxins (134). Two cDNAs were isolated and characterized. One of the cDNAs encoded toxin b, a short chain neurotoxin purified previously from the venom of Aipysurus laevis (135). The other cDNA encoded an isoform of toxin b, designated toxin d, not as yet described. Both cDNAs encoded precursor proteins with a 21-amino acid signal peptide and a 62-amino acid structural protein. The two amino acid sequences inferred from the nucleotide sequence were identical, except at positions 20, 25, and 28. At these positions toxin b was asparagine, methionine, and arginine; while toxin d was aspartic acid, lysine, and lysine, respectively.

Four species of African mamba snakes have venom neurotoxins that differ from other snake neurotoxins (37). Postjunctional alpha-neurotoxins, which bind to nicotinic cholinergic receptors, appear to be the only class of toxins mambas share with other snakes that have neurotoxic venoms. Typical mamba neurotoxins are the presynaptically-acting facilatatory toxins such as dendrotoxin, and anticholinesterase toxins such as fasciculin. Fasciculin and toxin C are proteins that inhibit the activity of acetylcholinesterase, an acetylcholine-hydrolyzing enzyme. A cDNA library was constructed by using mRNA isolated from the glands of the black mamba snake, Dendroaspis polylepis (136). Two clones isolated and characterized from the Dendroaspis polylepis cDNA library had inserts sizes of 430 base pairs (136). Both cDNAs had identical nucleotide sequences and encoded a precursor protein with a 21-amino acid signal peptide and a 61-amino acid structural protein of identical sequence to that obtained by direct sequence analysis (37).

Other snake toxin genes that have been cloned and characterized include myotoxin a from the prairie rattlesnake, Crotalus viridis viridis (137); crotamine isoforms from the South American rattlesnake, Crotalus durissus terrificus (138); and a thrombin-like enzyme, batroxobin, from the terciopelo snake, Bothrops atrox (139,140). Crotamine and myotoxin, appear to affect the functioning of voltage-sensitive, sodium channels of skeletal muscle sarcolemma, inducing a sodium influx resulting in a depolarization and contraction of skeletal muscle (141). In skeletal muscle, lesions from the effects of crotamine consist of necrosis of the muscle fibers characterized by extensive vacuolization of the sarcoplasmic reticulum and disruption of actin and myosin filaments (31). Two cDNA librar-

ies were constructed in an attempt to clone and characterize these toxin genes (137,138). One clone was isolated and characterized from the prairie rattlesnake cDNA library. This isolate had a cDNA encoding a precursor protein with a 22- amino acid signal peptide and a 43-amino acid polypeptide corresponding to the published sequence for myotoxin a (137), with the exception of an additional lysine on the C-terminus. This lysine, which is also present in the cDNAs encoding crotamine, is removed during post-translational processing. A bacteriophage cDNA library was constructed from mRNA isolated from the glands of the South American rattlesnake (138). The first high-density screening of 400,000 plaques for crotamine-containing genes yielded over 800 positives. Four of these clones with insert sizes from 270 to 400 base pairs were chosen and their inserts subcloned into pGEM-3Z and sequenced. The cDNAs analyzed encoded precursor proteins with a 22-amino acid signal peptide, a 42-amino acid structural protein, and a terminal lysine which was proteolytically removed. Nucleotide sequence analysis of the cloned cDNAs predicted the existence of multiple variants of the crotamine toxin. The different forms, identified from the DNA sequences, displayed discrepancies in amino acid sequence for crotamine when compared with previously published reports (142). Direct amino acid sequencing of commercially purified crotamine and CNBr fragments thereof confirmed the structures predicted by the nucleic acid sequences.

Precursors of secretory peptides are often synthesized as part of a large and inactive precursor protein, and contain sites for proteolysis and α-amidation. These sites are frequently marked by the sequence (-X-Gly-B-B) where X is the C-terminal amino acid residue in the mature peptide that is α-amidated, and B is either lysine or arginine (78,79). As discussed below, cDNAs have been isolated from an Androctonus australis Hector cDNA library that encode precursor proteins for the scorpion toxin II (AaH II). The inferred amino acid sequence of the precursor toxin contains two additional terminal residues, Gly-Arg, not found in the mature toxin. The post-translational removal of the dipeptide, Gly-Arg, in the pre-protein, and the subsequent α-amidation of its terminal histidine residue (73,143,144) are modifications previously observed with other α-amidated proteins (78,79). The only difference in this case of the AaH II is that it has one additional basic residue instead of the usual two or none at all, as in the case of mellitin from bee venom (145). Interestingly, the terminal amino acid in myotoxin a and crotamine is glycine and the precursor proteins have the terminal sequence X-Ser-Gly-Lys. However, in the

snake it is the lysine that is removed and not the Gly-Lys, leaving an α-amidated serine. Perhaps snakes have lost the specific enzymes required for this modification but still retain the amino acid sequence that acts as the substrate for the processing enzymes. Future experiments in which these cDNAs are expressed in a system such as baculovirus, in which α-amidation occurs, will be interesting.

The gene encoding batroxobin, a thrombin-like enzyme, has been cloned and characterized at both the cDNA and the genomic level (139,140). The cDNA encodes a precursor protein with a signal peptide of 24-amino acids followed by a 231-amino acid protein. The nucleotide sequence from the genomic clone demonstrates that the batroxobin gene consists of five exons and four introns that encode the mature batroxobin. The total length of this gene is 8.0 Kb, 6.0 Kb of which comprise intron regions. The nucleotide sequence of the gene indicated that batroxobin is a member of the trypsin/kallikrein family rather than the prothrombin family.

Toxin genes from animal sources other than snakes have also been cloned and their genes sequenced. Gene libraries have been prepared from mRNA isolated from the North African fat-tailed scorpion (Androctonus australis Hector)(143,144), the cloth-of-gold cone snail (Conus textile) (146), the honey bee (Apis mellifera)(147,148), and the black widow spider (Latrodectus mactans (150). With mRNA isolated from the telsons of Androctonus australis Hector, a cDNA library was constructed by using the Okayama/Berg cloning strategy described earlier. Full-length cDNAs of about 370 nucleotides encoding precursors of toxins active on mammals or on insects were isolated and sequenced. Sequence analysis of the cDNAs reveal the precursors contained signal peptides of 19 amino acid residues for the mammal toxins and 18 residues for the insect toxins. In addition, precursors of toxins active on mammals have extensions on their C-terminal ends, Arg or Gly-Arg (144), while those active in insects did not possess these extensions. The extensions are removed during post-translational processing and the removal of the dipeptide, Gly-Arg, results in the terminal amino acid becoming α-amidated. Clones isolated and characterized from this library included those encoding for mammal toxins AaH I, AaH I', AaH II, and AaH III, and insect toxins AaH IT1, and AaH IT2. The insect toxin cDNAs encoded mature toxins of 70 amino acid residues while those for the mammal toxins encoded 63 amino acid residues for AaH I and AaH I' and 64 amino acid residues for AaH II and AaH III. Only the mammal toxin AaH II was α-amidated on the C-terminus.

A Conus textile cDNA library was also constructed by using an Okayama/Berg vector and mRNA isolated from venom ducts of the cone snail

(146). The library was screened for clones harboring cDNAs encoding the conotoxin designated as the King Kong peptide (149). The peptide was named on the basis of a behavioral modification it induces in lobsters (149). Nucleotide sequence analysis of cloned cDNAs revealed a family of King-Kong related toxin transcripts. Three different pro-peptide cDNA sequences were obtained; only one of these encoded sequence for the King-Kong peptide. The other cDNA sequences encoded two different peptides designated as KK-1 and KK-2. When the predicted pro-peptide sequences are compared, well defined conserved and hypervariable regions can be identified. The hypervariable regions comprise four regions between cysteine residues in the final peptide toxin. Conserved sequences include the disulfide bonded Cys residues found in the mature toxin and the N-terminal regions of the pro-peptide, which are excised during post-translational processing. It is the conserved regions that may direct the formation of a specific disulfide configuration in the King-Kong family of conotoxins (146).

The final section will review work on the expression of animal toxin genes. There have been several reports of animal toxins (151,152,153) and non-toxic PLA2 (154) expressed in Escherichia coli. Two synthetic genes were constructed encoding the two toxins, echistatin (151) and neurotoxin B-IV (152). Echistatin is a potent platelet aggregation inhibitor (PAI) purified from the venom of the saw-scaled viper, Echis carinatus. It is a protein of 49-amino acids containing eight cysteine residues (56). Treatment of the toxin with reducing agents abolishes its PAI activity (56, 151). Neurotoxin B-IV can be isolated from the mucus secretions of the anoplan nemertine, Cerebratulus lacteus. Neurotoxin B-IV prolongs the repolarization phase of the action potential in crustacean nerve via interaction with voltage-sensitive sodium channels, but is essentially nontoxic to other classes or phyla. Neurotoxin B-IV is a 55-amino acid polypeptide containing four disulfide bonds (155).

The chemically synthesized gene for [Leu-28]echistatin was inserted into an E. coli expression vector. The gene was inserted as a fusion protein, later to be separated from its carrier by CNBr cleavage. A methionine residue, normally present at position 28 in the native protein, was replaced by leucine so that the expressed protein would not be degraded during the CNBr step. The expression vector was constructed by inserting portions of the E. coli *cheB* and *cheY* gene complex into the plasmid pUC13 (156). High-level expression of the synthetic toxin was achieved by its fusion with the E. coli *cheY* gene and the recombinant product was liberated from the fusion protein by CNBr cleavage. After a renaturation

step was performed on the released recombinant, the protein was purified by reverse-phase chromatography. The amount of correctly folded, pure [Leu-28]echistatin obtained was estimated to be about 1.5 mg per liter of cell culture. The refolded protein was identical to the native echistatin in inhibiting platelet aggregation. Since 1 g of lyophilized E. carinatus venom yields about 1-2 mg of pure echistatin, molecular biology has indeed been used to produce potentially larger yields of protein without the added danger in handling such a dangerous snake.

The chemically synthesized gene for the neurotoxin B-IV was also cloned and expressed as a fusion protein with either E. coli β-galactosidase or the gene 9 protein of bacteriophage T7 (152). The fusion protein was purified and released from its fusion-escort by Factor Xa-catalyzed hydrolysis at a customized linker site. The yield of purified recombinant protein from 1 liter of culture was 12 mg. The recombinant protein was identical to the neurotoxin B-IV isolated from Cerebratulus with respect to its mobility on high pressure liquid chromatography and its secondary structure, as determined by circular dichroism. Although the recombinant product had an additional methionine at the amino-terminus and a replacement of proline for hydroxyproline at position 10, the specific toxicity determined by bioassay was comparable to the native toxin.

A postsynaptically acting snake venom neurotoxin (153) and a non-toxic, porcine pancreatic phospholipase A2 (154) have also been expressed in E. coli using genes from cDNAs. Both of these genes were inserted also into E. coli expression vectors as fusion proteins. In the case of the non-toxic PLA2, cleavage of the fused protein was accomplished by using hydroxylamine or trypsin, and renaturation of the recombinant protein was accomplished by using a S-sulfonation method (157). The purified recombinant protein had yields of 2-3 mg per liter of cell culture and displayed identical properties compared to the native protein (154). A cDNA encoding the postsynaptic neurotoxin, erabutoxin a, was fused to commercially-available expression vector, pRIT5 (153). This vector was designed to permit high-level expression of fusion proteins in both E. coli and Staphylococcus aureus cells (158). Foreign genes, inserted into a multiple cloning site, are fused to a vector sequence encoding a 31-Kd protein A moiety. Foreign genes can then be expressed from the protein A promoter and can be translocated to the periplasmic space by E. coli, or secreted into the growth media by S. aureus. This system has an additional feature in that the IgG-binding domain of protein A can provide a rapid method for purifying the fusion protein by an IgG Sepharose 6 FF affinity column. Thus, the construct was originally designed so that the recombinant protein would

be released from the fusion protein with CNBr treatment. Interestingly enough, however, the fusion product itself was produced in a correctly folded conformation and was directly secreted as such into the periplasmic space of E. coli. Competitive binding experiments showed the hybrid protein blocked erabutoxin a binding to the acetylcholine receptor and to a monoclonal antibody that recognized short-chain neurotoxins. In addition, the fusion protein was not only more immunogenic and less toxic than the native erabutoxin a, it was capable also of inducing neutralizing antibodies as potent as those raised against the native toxin (153). This approach may be extremely useful for future development of serotherapy against envenomation by poisonous animals.

Expression of scorpion toxin genes has been attempted in monkey kidney COS cells (143,144), baculovirus (159), and in NIH/3T3 mouse fibroblast cells (160). One of the cDNAs, designated pcD403, encoding the mammal toxin II from Androctonus australis Hector was used to transfect COS-7 (SV40-transformed African green kidney monkey) cells. The recombinant AaH II, expressed and secreted by the COS-7 cells, was characterized by an immunoassay, a receptor-binding assay, and a bioassay. In all three methods, the recombinant product behaved identically to native AaH II. The results obtained support the conclusion that the recombinant toxin monkey kidney cells transiently expressed and secreted upon their transfection with the recombinant plasmid, pcD403, was the mature form of AaH II. The yield of secreted product from expression in this system was 0.2 μg of affinity-purified recombinant per 10^6 cells (143,144).

Baculovirus was used to express a synthetic insect-specific neurotoxin gene (159). A 112-base pair gene (*Belt*) encoding the insectotoxin-1, isolated from the middle-Asian subspecies of the scorpion Buthus eupeus, was synthesized, inserted into a transplacement or shuttle vector, and cloned in E. coli. The gene was transferred to the baculovirus genome of Autographa californica nuclear polyhedrosis virus (*Ac* MNPV) by homologous recombination. Three different recombinant *Ac* MNPVs, carrying *Belt* under the control of the strong *Ac* MNPV polyhedrin promoter, were constructed and used to infect Spodoptera frugiperda (Sf9) ovary insect cells in tissue culture. The highest level of expression was detected in the construct in which a fusion gene comprised of 58 codons corresponding to the N-terminal sequence of the polyhedrin was fused to *Belt*. Although the polyhedrin promoter-directed transcripts of all three constructs accumulated to levels observed for wild-type virus, the highest expressed recombinant protein was 10- to 20-fold less than that for polyhedrin. This result indicated that the impediment to expression was at the level of

translation and product stability rather than at the transcriptional level. The neurotoxicity associated with the native toxin was not detected in the fused recombinant protein, presumably due to the 58 additional amino acids fused to the expressed toxin.

Finally, cultured murine cells were used to express a synthetically-constructed scorpion neurotoxin (160). The scorpion toxin gene (AaIT) encoded the insect-specific toxin I from Androctonus australis, which had been characterized previously (161). The synthetic gene was inserted into the pAC380 plasmid (162), and a leader sequence encoding the signal peptide of human interleukin-2 (IL2-SP) was fused to the insect toxin gene. This recombinant construct (IL2-SP-AaIT) was subsequently cloned into the prokaryotic expression vector, pL47 (S.R. Jaskunas, Lilly Research Laboratories), and then into the plasmid pMSV (163), which contains the entire proviral sequence of Moloney murine sarcoma virus (Mo.MSV). The resulting plasmid, designated pMSV-IT, placed the expression of the AaIT gene under the transcriptional control of the Mo.MSV long terminal repeats (LTRs). The recombinant plasmid, pMSV-IT, was then used to infect NIH/3T3 mouse fibroblast cells. The toxin gene, when placed under the transcriptional control of the Mo.MSV LTRs, was expressed in a biologically active form functionally indistinguishable from its natural counterpart (160).

CONCLUSION

The application of molecular biology to the study of animal toxins and their genes is entering a phase of rapid development. This technology will undoubtedly play an important role in many areas of animal toxin research in the future. Some of these areas will include studies on toxin structure and the relationship to activity and function; generation of recombinant toxins and their genetically-mutated derivatives for use in antibody production and vaccine development; and in basic research studies elucidating the organization of genes and the regulation of their expression in venomous animals.

The ability to express toxin genes in heterologous host systems holds the key to realizing the full potential of recombinant DNA technology as applied to animal toxin research and vaccine development. The structural complexity of these proteins and the fact that these are toxic to certain cells, can make their expression difficult. As we have seen, fusion proteins can be effective in reducing the lethal potency of some toxins, while still retaining or even increasing their immunogenic ability for producing

neutralizing antibodies against the active toxin. In these cases, fused toxins will be extremely valuable for future development of serotherapy against envenomation. However, there may be many situations where fused toxins will not elicit neutralizing antibodies. In those cases, further work must be performed, either at the level of the expression system being used, or in the manipulation of the fused toxin in the removal of its escort or fused carrier.

One of the celebrated features of recombinant DNA technology is the wide array of techniques, expression vectors and hosts to choose from. Recently, it was said, "Expression systems are protein specific. You must be able to play around with each one, insert your gene of choice, tweak it, and then see what you've got" (164). This area of research is exciting, challenging, and holds much promise for future progress towards developing protective modalities against toxins. I hope this review will be an enticement for others to learn and apply recombinant DNA technology to the study of animal toxins and toxin genes.

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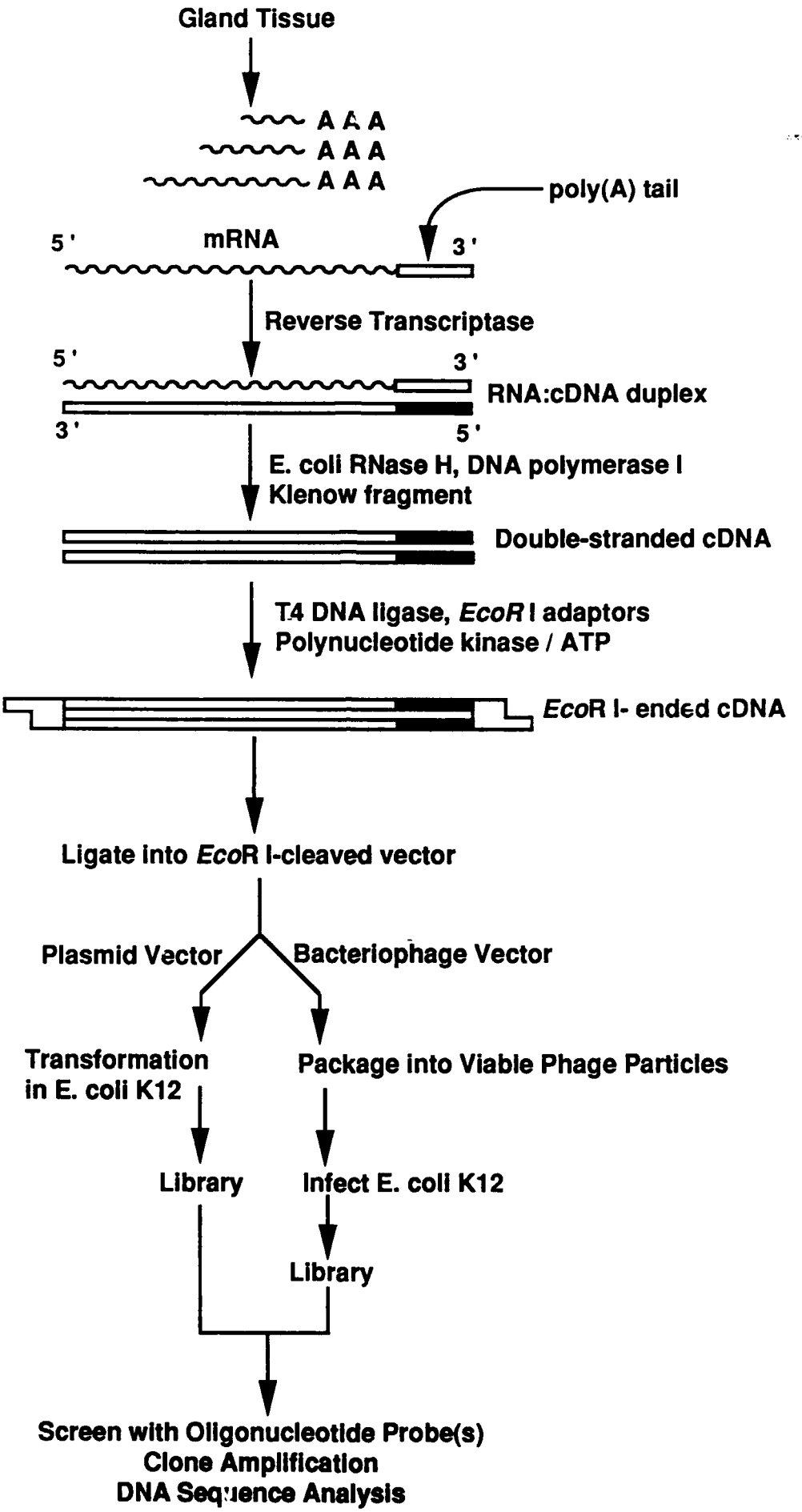
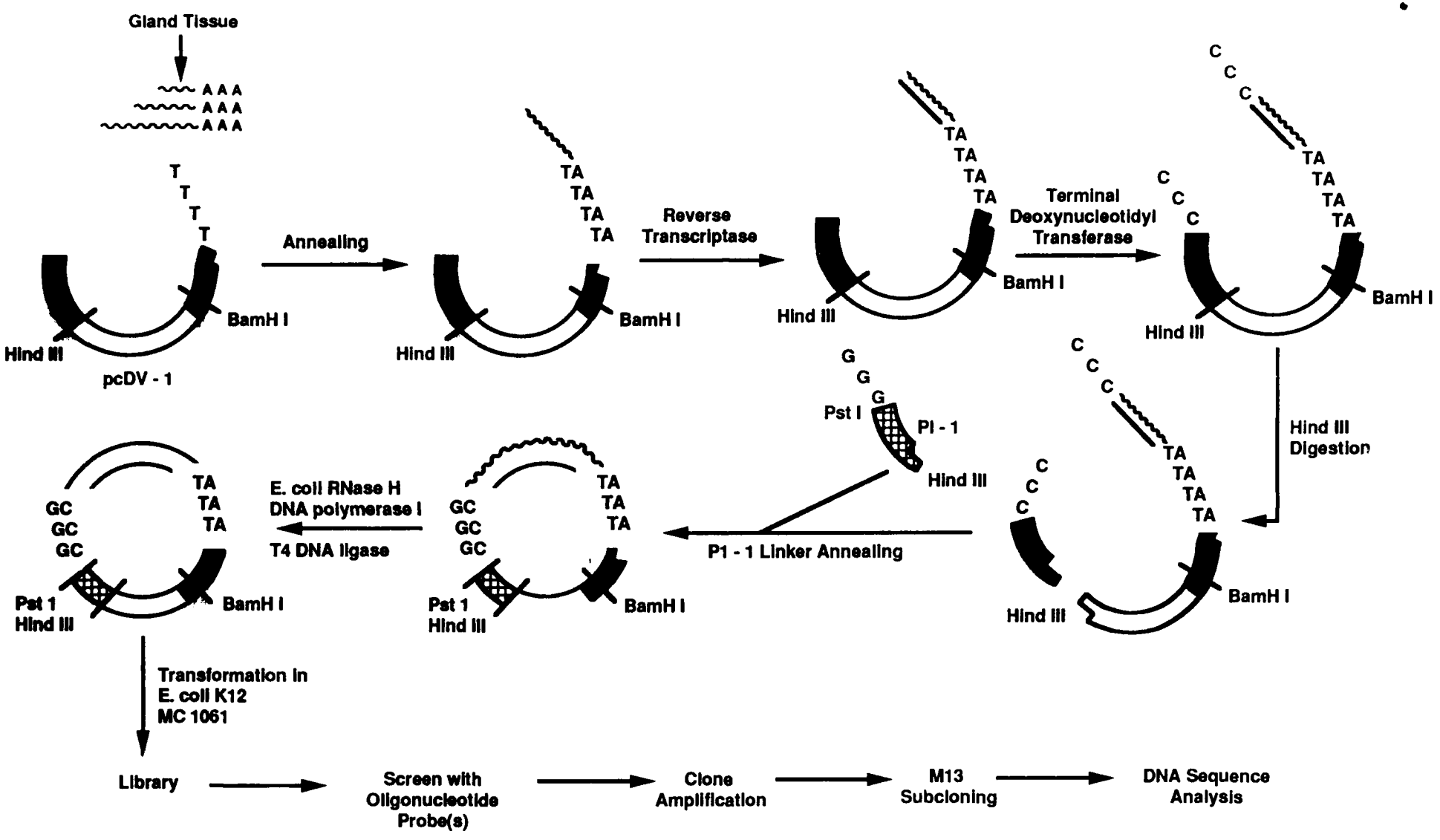


Figure 1A. cDNA Cloning Strategy



Okayama, H. and Berg, P. (1983) *Mol. Cell. Biol. 3*, 280.

Figure 1B. cDNA CLONING STRATEGY - The Okayama / Berg System

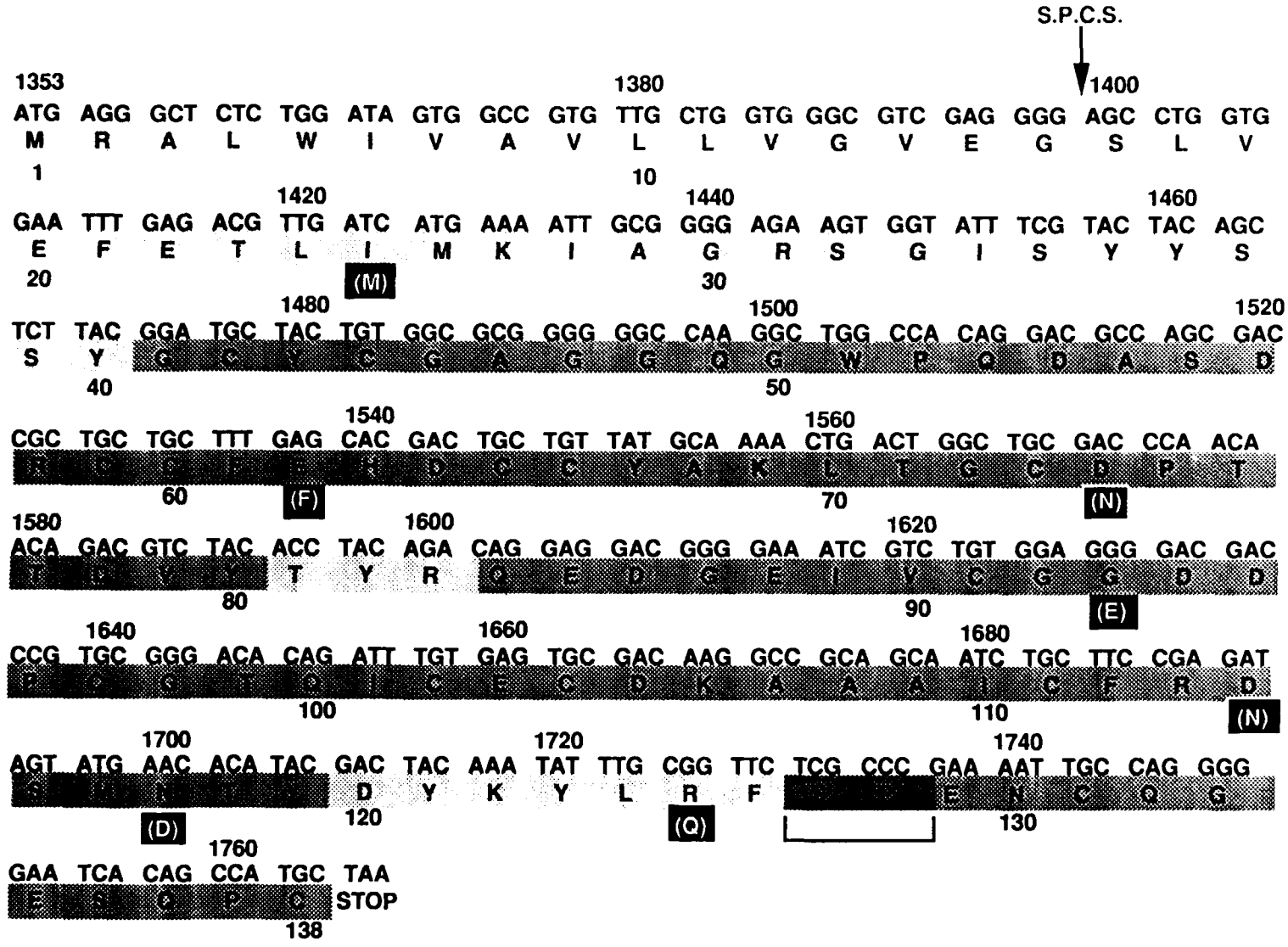


Figure 2. Nucleotide and Inferred Amino Acid Sequence of Mojave Toxin Acidic-Subunit Gene